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Ganglion Cells Are Required for Normal Progenitor-Cell Proliferation but Not Cell-Fate Determination or Patterning in the Developing Mouse Retina

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Summary

The vertebrate retina develops from an amorphous sheet of dividing retinal progenitor cells (RPCs) through a sequential process that culminates in an exquisitely patterned neural tissue [1]. A current model for retinal development posits that sequential cell-type differentiation is the result of changes in the intrinsic competence state of multipotent RPCs as they advance in time and that the intrinsic changes are influenced by continuous changes in the extracellular environment [2]. Although several studies support the proposition that newly differentiated cells alter the extrinsic state of the developing retina [2, 3], it is still far from clear what role they play in modifying the extracellular environment and in influencing the properties of RPCs. Here, we specifically ablate retinal ganglion cells (RGCs) as they differentiate, and we determine the impact of RGC absence on retinal development. We find that RGCs are not essential for changing the competence of RPCs, but they are necessary for maintaining sufficient numbers of RPCs by regulating cell proliferation via growth factors. Intrinsic rather than extrinsic factors are likely to play the critical roles in determining retinal cell fate.

Results

Specific and Efficient Ablation of RGCs

To specifically ablate RGCs, we used a binary system to express diphtheria toxin A (*dta*) in newly formed RGCs. We inserted a *floxed-lacZ-stop-dta* cassette [4] into the *brn3b* locus to create the *brn3b*$^{Z\text{-}dta}$ allele (see Figure S1A in the Supplemental Data available with this article online). Excision of *lacZ* by Cre recombinase restores the open reading frame of *dta* [4]. *Brn3b* is one of the earliest genes expressed in the RGCs immediately after their birth [5, 6]; *lacZ* expression from the *brn3b*$^{Z\text{-}dta}$ allele recapitulated the *brn3b* expression pattern and was restricted solely to the RGCs in the retina at E12.5 and E14.5 (Figure S1B; Figure 1A).

Mice harboring one copy of *brn3b*$^{Z\text{-}dta}$ were bred to a *six3-cre* transgenic line [7]. Because *brn3b* and the *six3-cre* transgene are expressed in nonoverlapping patterns except in RGCs (Figure S1C; [5–7]), the *brn3b*$^{Z\text{-}dta/+}$*;six3-cre* mice will produce active DTA only in RGCs. The Cre recombinase from the *six3-cre* transgene efficiently and specifically deleted *lacZ* from the *brn3b*$^{Z\text{-}dta}$ allele, as determined by PCR genotyping (Figure S1D). Whereas both *lacZ* and *dta* sequences were detected in retinas from *brn3b*$^{Z\text{-}dta/+}$ embryos, only *dta* sequences were present in retinas from *brn3b*$^{Z\text{-}dta/+}$*;six3-cre* embryos (Figure S1D). This was further confirmed by *lacZ* staining. In contrast to *brn3b*$^{Z\text{-}dta/+}$ controls, *lacZ* was undetectable in *brn3b*$^{Z\text{-}dta/+}$*;six3-cre* retinas (Figure 1A).

At E14.5, *brn3b*$^{Z\text{-}dta/+}$*;six3-cre* retinas were notably thinner, and the optic disc was deformed (Figure 1A), indicating that RGCs had been ablated. By examining the expression of RGC-specific genes [8], including *NF66*, *persyn*, and *brn3b* (Figure 1A), we estimated that by E14.5, more than 98% of the RGCs were ablated in the *brn3b*$^{Z\text{-}dta/+}$*;six3-cre* retinas. The very few remaining RGCs were mostly migrating, immature RGCs. By E17.5, virtually no *brn3b*-positive cells were detected (data not shown). The RGCs remaining at E14.5 were therefore likely to exert minimal, if any, non-cell-autonomous effects because of their small numbers and immature state of differentiation.

Morphological and Electrophysiological Changes in RGC-Ablated Mature Retinas and Optic Nerves

The optic nerves from P16 retinas where RGCs were ablated were clearly abnormal; they were substantially thinner than controls (Figure 1B) and exhibited small-diameter fibers that did not resemble normal RGC axons (Figure 1C). In addition, these fibers were not myelinated, although there were layered myelin-like structures that did not circumscribe the residual nerve fibers (Figure 1C).

To determine whether RGC depletion led to observable physiological defects, we recorded electroretinogram (ERG) responses in adult RGC-depleted mice. *Brn3b*$^{Z\text{-}dta/+}$ mice had normal ERGs [9], but under both dark- and light-adapted conditions, *brn3b*$^{Z\text{-}dta/+}$*;six3-cre* mice had severe abnormalities (Figures 1D and 1E). Specifically, the saturated amplitudes of the dark- and light-adapted b-waves in RGC-depleted mice were 25% of the control amplitudes, and the saturated dark-adapted a-wave amplitudes were 42% of controls. The negative scotopic threshold response (nSRT) was very small in all RGC-depleted mice, and the positive SRT (pSRT) was absent (Figure 1E). Thus, ablation of RGCs during development affected gross functional measures of not only RGCs, but also photoreceptor cells and intermediate neurons within the inner nuclear layer [10, 11].

Consistently, we observed that RGC-ablated P16 ret-

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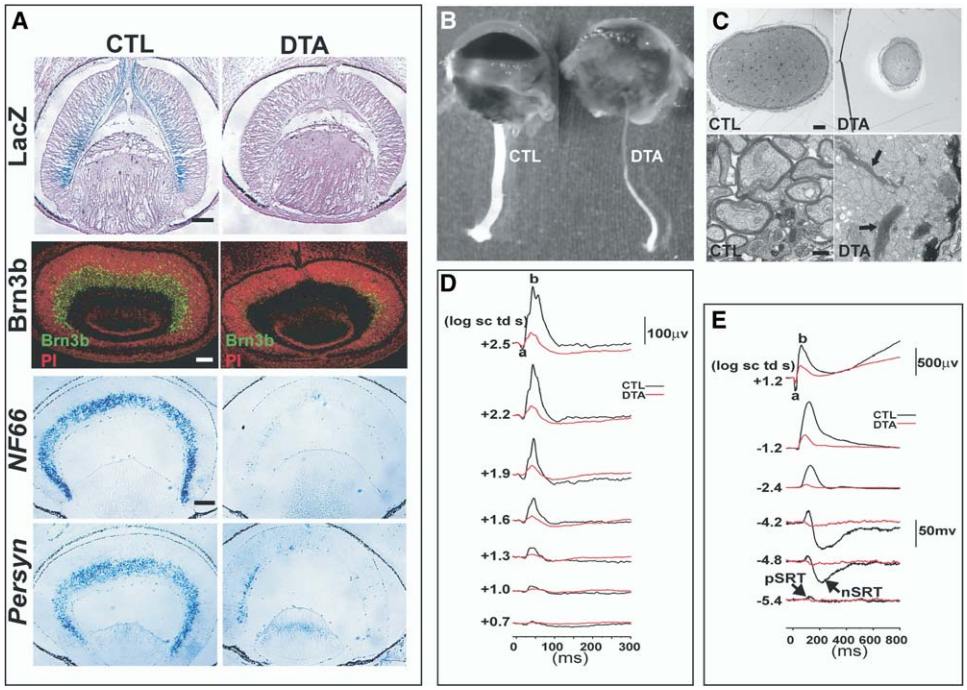

Figure 1. The Binary Transgenic System Effectively Ablates RGCs and Results in Abnormal Mature Retinas

(A) X-gal staining for lacZ, immunofluorescence staining for Brn3b (green; red is nuclear staining), and in situ hybridization for *NF66* and *Persyn* of control *brn3b*$^{Z\text{-}dta/+}$ (CTL) and *brn3b*$^{Z\text{-}dta/+}$*;six3-cre* (DTA) retina sections. In comparison to the control, in which all these markers were expressed in the ganglion cell layer, little signal was detected for any of them in the *brn3b*$^{Z\text{-}dta/+}$*;six3-cre* (DTA) retina, suggesting that the RGCs were efficiently ablated. The scale bars represent 100 μm.

(B and C) Abnormal optic nerve of RGC-ablated P16 mice. (B) Dissected eyeballs and optic nerves from control (CTL) and *brn3b-dta*-activated (DTA) mice are shown. (C) TEM images of cross-sections from control (CTL) and *brn3b-dta*-activated (DTA) mice are shown. The top panels are shown at 400× magnification (the scale bar represents 40 μm), and bottom panels at 20,000× (the scale bar represents 500 nm). Arrows indicate myelin-like structure in the RGC-ablated optic nerve.

(D and E) ERGs of littermate 2-month-old control mice (CTL) and mice with RGC-ablated retinas (DTA) under light- (D) and dark-adapted (E) conditions of varying strength. X axes are time (ms) lapsed after flash. Stimulus energy is indicated (log scotopic Troland s). nSRT denotes negative scotopic threshold response, and pSRT denotes positive scotopic threshold response.

inas were 30%–50% thinner than controls. This reduction in thickness was uniform across the whole retina (Figure 2A). There were significantly fewer cells in all three layers of ablated retinas than in control retinas; approximately 40% of the cells were missing from each layer (Figure 2B). The residual cells present in the ganglion cell layer were displaced amacrine cells. The results suggested that there was an overall reduction of differentiated cells in RGC-ablated retina.

Differentiation of Other Cell Types Does Not Require RGCs

At P16, all cell types, with the exception of RGCs, were present in the RGC-ablated retinas, although their absolute numbers were reduced in all layers (Figure 2C). Brn3b-positive cells were absent in these retinas, as expected (Figure 2C). Pax6, a marker for amacrine cells, was expressed within the ganglion cell- and inner nuclear layer, and syntaxin expression, which indicated the presence of amacrine cell processes, appeared in the inner plexiform layer (Figure 2C). Similarly, PKCα, a marker for rod bipolar cells, was expressed in the expected pattern (Figure 2C). Chx10, a general bipolar cell marker, and NF160, a marker of horizontal cells, were also expressed in normal patterns (Figure 2C). NF160 is also expressed in RGCs, and its expression was absent in the ganglion cell layer of RGC-depleted retina. Rhodopsin-positive cells (Rods) and cone arrestin (CAR)-positive cells (Cones) [12] were found in the outer nuclear layer (Figure 2C). As identified by vimentin and cellular retinaldehyde binding protein (CRALBP) expression, Müller glial cells, the last retinal cells to differentiate, were distributed in their normal pattern with their nuclei in the inner nuclear layer and processes extending into the inner and outer plexiform layers (Figure 2C). Quantification of the different cell types in the whole retinal population revealed no significant proportional change in any of the cell types (Figure 2D). Together, these results strongly suggest that depletion of RGCs did not affect the normal specification and differentiation of non-RGC cell types. Moreover, the overall integrity of the retina was maintained; we saw no evidence of disrupted morphology in RGC-ablated retinas as late as 5 months after birth (data not shown).

Reduced Cell Number in Mature RGC-Ablated Retinas Results from Decreased RPC Proliferation but Not Enhanced Apoptosis

The cell number reduction consequent to RGC ablation could have been caused by decreased RPC prolifera-

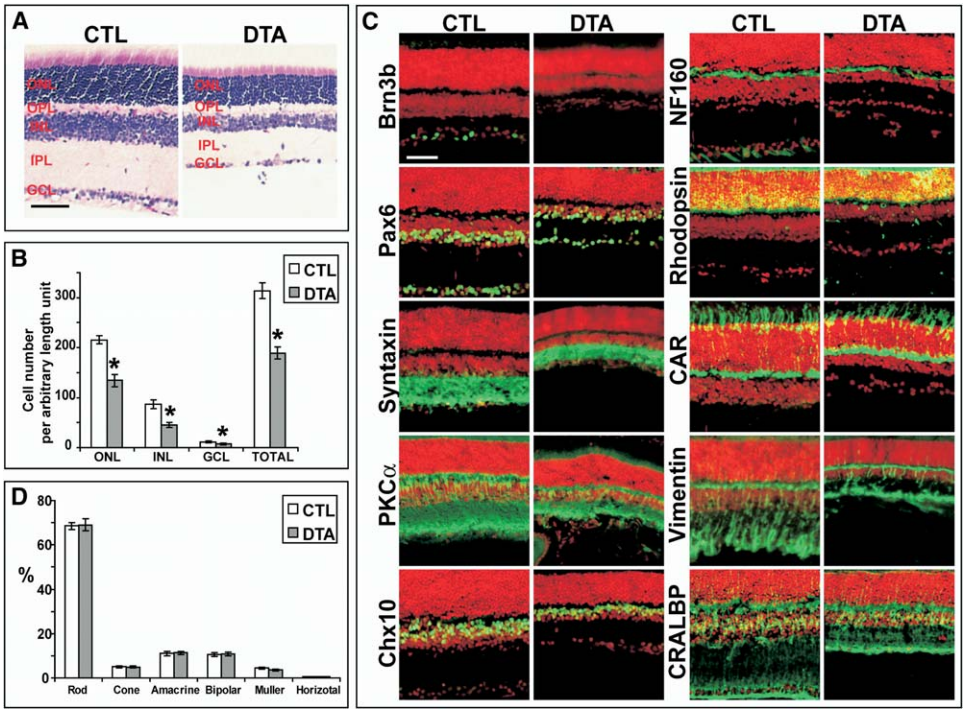

Figure 2. RGC-Ablated Retinas from P16 Mice Are Thinner and Have Fewer Cells in All Three Nuclear Layers but Contain All the Other Cell Types

(A) Hematoxylin and Eosin (H&E) staining of sections of control (CTL, *brn3b*$^{Z\text{-}dta/+}$) and RGC-ablated (DTA) retinas (P16). The following abbreviations were used: ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; and GCL, ganglion cell layer. The scale bar represents 50 μm.

(B) Cell numbers in nuclear layers of RGC-ablated retinas. A total of 5 arbitrary length units from 5 different sections were counted for each genotype in the central retina region. Error bars indicate the standard deviation from the mean. * indicates $p < 0.001$.

(C) Expression of retinal cell-type-specific markers in control (CTL) and RGC-ablated (DTA) P16 retinas by fluorescence labeling. Gene expression markers (green) are indicated on the left of each image. Nuclei (red) were stained with propidium iodide. The scale bar represents 30 μm.

(D) Quantification of different cell types in the control (CTL) and RGC-ablated (DTA) retinas. For each cell type, marker-positive cells and total cells (on the basis of propidium iodide staining) were counted within an arbitrary length unit in the central retina region of stained P16 sections, and the percentage was calculated. The y axis is the average percentage of each cell type from eight sections from two animals of each genotype. Error bars indicate the standard deviation from the mean.

tion, enhanced apoptosis, or both. To measure proliferation, we used BrdU to chase-label S phase RPCs at E14.5. There were substantially fewer dividing RPCs in the proliferation zone of RGC-ablated retinas than in the controls (Figure 3A). The reduction was most pronounced in the central retina region, particularly on the apical side, producing a narrower zone of S phase cells (Figure 3A). There were 38% fewer BrdU-positive cells in RGC-ablated retinas than in controls (Figure 3B). The decreased proliferation may have resulted from either a reduction in cell number or a prolonged cell cycle. Although we cannot distinguish between these possibilities, a similar reduction of dividing RPCs was also observed at E17.5 (data not shown). It is possible that RGCs were continuously required for efficient RPC proliferation at later stages or that inefficient proliferation at an early stage led to a smaller RPC pool.

We used anti-active caspase 3 to detect apoptotic cells in E14.5 RGC-ablated and control retinas and a Brn3b antibody to distinguish RGCs from other retinal cells. We detected little apoptosis in control retinas from *brn3b*$^{Z\text{-}dta/+}$ embryos (Figure 3C). In *dta*-activated retinas, although there were a substantial number of active caspase-3-positive cells in the RGC-ablated retinas, these cells were located near the retinas' basal side, where differentiated RGCs would normally reside (Figure 3C). Because DTA cytotoxicity induced apoptosis [13], the apoptotic cells in this region almost certainly represented dying RGCs (Figure 3C). Most important, we saw no apoptotic cells in the proliferation zone, even though some Brn3b-positive cells were seen there (Figure 3C). The Brn3b-positive cells were newly born, immature RGCs, and there seemed to be a lapse between the onset of Brn3b expression and DTA-mediated RGC cell death. Decreased RPC proliferation, not enhanced apoptosis, was responsible for the reduced cell number observed in the mature RGC-ablated retina.

Changes in Gene Expression in RGC-Ablated Retinas

Mutations in the genes encoding Chx10 and cyclin D1 cause severe proliferation defects in the retina [14, 15]. Both genes are expressed in proliferating RPCs, but not in postmitotic RGCs [14, 15]. Chx10 protein production was substantially reduced in RGC-ablated retinas from

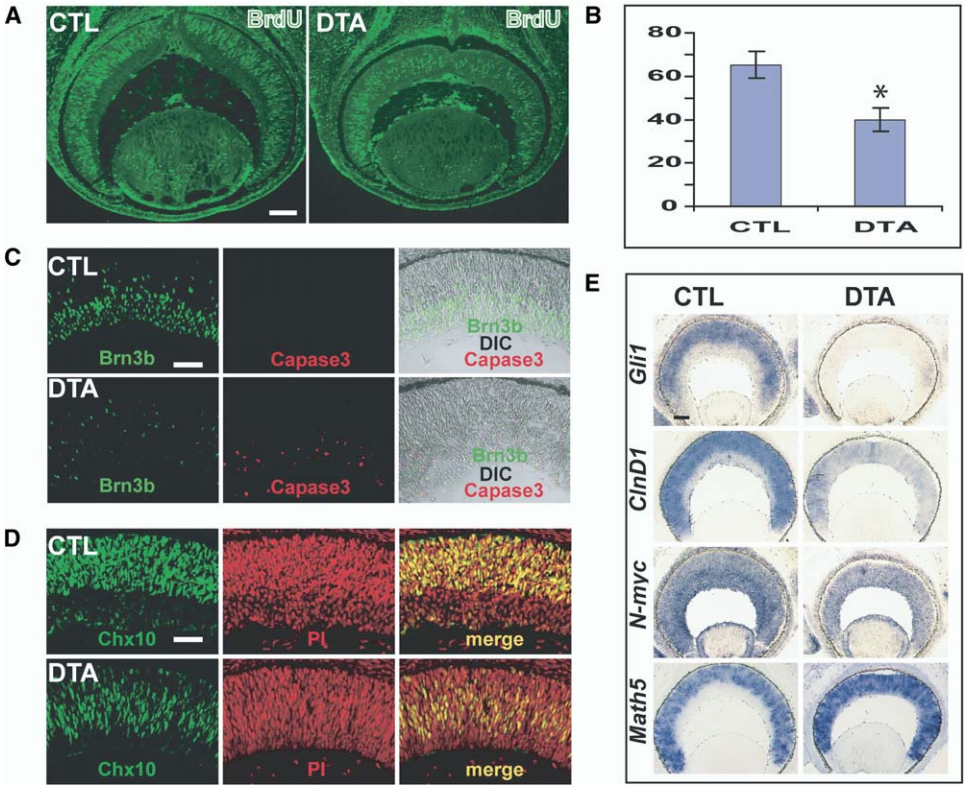

Figure 3. RPC Proliferation and Apoptosis in Control (CTL, *brn3b*$^{Z\text{-}dta/+}$) and RGC-Ablated (DTA, *brn3b*$^{Z\text{-}dta/+}$;*six3-cre*) E14.5 Retinas

(A) BrdU labeling (green) of control (left) and RGC-ablated (right) retinal sections. Note that very few cells on the apical side of the proliferation zone were BrdU positive.

(B) Number of BrdU-positive cells in control and RGC-ablated retinas per arbitrary length unit in the central retina region. n = 8. * indicates $p < 0.001$.

(C) Anti-active caspase 3 and anti-Brn3b antibody staining to detect apoptotic (caspase-positive, red) cells and RGCs (Brn3b-positive, green) from control and RGC-ablated E14.5 retinas. All the apoptotic cells in the RGC-ablated retina were located close to the basal side.

(D) Immunofluorescence labeling of Chx10 (Green). Red indicates nuclear staining by propidium iodide. Chx10 staining was reduced in the RGC-ablated retina. This was more obvious on the apical side.

(E) In situ hybridization for *Gli1*, *cyclinD1(ClnD1)*, *N-myc*, and *math5*. In (A), the scale bars represent 100 μm; in (C) and (D), 50 μm; and in (E), 100 μm.

E14.5 embryos (Figure 3D). The reduction was more severe on the apical side of the proliferation zone (Figure 3D). *cyclin D1* transcript expression also was substantially reduced in RPCs of RGC-ablated retinas (Figure 3E).

RGCs could influence RPC proliferation by expressing genes encoding secreted signaling molecules. At E14.5, Sonic hedgehog (Shh) is expressed in RGCs, and Gli1, a downstream transcription factor, is expressed in the overlying RPCs [8, 16]. *Gli1* expression is dependent on Shh secreted from RGCs [8, 16]. The absence of RGCs caused a complete loss of *Gli1* expression (Figure 3E), providing further evidence that RGCs are a major source of secreted Shh at this stage in retinogenesis. In other neural tissues, Shh signaling regulates *cyclin D1* expression through the action of N-myc [17]. This did not seem to be the case in the retina because no significant change of *N-myc* expression in the proliferation zone was observed in the RGC-ablated retina (Figure 3E).

math5 is a proneural bHLH transcription factor gene crucial for the formation of RGCs [18, 19] and is expressed in a subset of RPCs in the proliferation zone before they commit to an RGC fate ([20]; Figure 3E). In RGC-ablated retinas, we observed a reproducible upregulation of *math5* expression in RPCs (Figure 3E). These results suggested that RGC ablation led to a larger pool of RGC-competent progenitor cells.

Discussion

Role of RGCs in the Development of Other Retinal Cell Types

During retinogenesis, early born cell types regulate their numbers by negative feedback mechanisms [21], but it is not clear how they influence the competence state of RPCs. RGCs secrete various signaling molecules [8, 16, 21, 22] that could potentially function in cell-fate decisions. Ablation of RGCs as they differentiate essentially eliminated these extrinsic signals. Because RGC removal did not affect the specification of any of the other six cell types, the transition of RPCs from one competence state to another is not caused by environmental changes stemming from RGC differ-

entiation. This is consistent with a recent report suggesting that extrinsic signals play relatively minor roles in the competence changes of RPCs during development [23] and with results from gene knockouts in which one or more retinal cell types are missing but other cell types still develop normally [18, 19]. Although the mechanism for the progressive change in RPC competence remains unclear, it is apparent that intrinsic factors, rather than extrinsic factors, play the major roles.

RGCs Control Normal RPC Proliferation by Extrinsic Signaling from Shh and Other Signaling Molecules

RGCs clearly regulate RPC proliferation, and this is likely to be achieved by influencing the expression of genes that regulate the cell cycle, in particular *cyclin D1* and *Chx10* [14, 24]. Cell-cycle progression may be mediated by extrinsic factors, and because Shh is a potent mitogen for RPCs [25], a plausible hypothesis is that the effects of RGCs on RPC proliferation are a consequence of Shh signaling through *Gli1*. Gli1 may directly control *cyclin D1* expression [26], or it may function through intermediate factors [17].

RGC-specific expression of *Shh* is highly dependent on Brn3b [8], a factor that is required for RGC differentiation [5, 27]. This dependence indicates that a regulatory pathway from Brn3b to Shh to Gli1 operates to maintain RPCs in a state of optimal proliferation.

Other than promoting RPC proliferation, Shh also negatively regulates RGC production through a feedback mechanism [21]. Although *Shh* expression is severely downregulated in *brn3b* null and RGC-ablated retinas, we obviously would not be able to detect increases in RGC number because RGCs do not survive. Nevertheless, *math5* was upregulated in the absence of RGCs, indicating that a larger number of RPCs were adopting an RGC fate. We did not observe defects in retinal organization in either *brn3b* null or RGC-ablated retinas, as has been reported for *Shh* null retinas [16]. The discrepancy might be explained by the possibility that later in development in the mouse, as in the case for zebrafish [28], *Shh* expression is not restricted to RGCs.

Besides Shh, two other secreted molecules, myostatin/GDF8 and VEGF, directly associated with neuronal progenitor cell proliferation are produced by differentiated RGCs [8, 22]. They could also participate in regulating the rate of cell proliferation in RPCs.

Comparison of RGC-Ablated Retinas with Retinas from *brn3b*- and *math5*-Knockout Mice

A thin-retina phenotype was also observed for the *brn3b* null mice [27], and there appear to be fewer cells in all three nuclear layers of *brn3b* null retinas [27]. Earlier studies did not address the basis of this phenotype, but in light of the results presented here, RGC defects in *brn3b* null retinas during development may lead to a RPC-proliferation-rate reduction similar to that seen in RGC-ablated retinas.

Retinas from *math5* null mice also exhibit a thin-retina phenotype, but the defect is not uniform across the nuclear layers; the inner nuclear layer is most affected, whereas the photoreceptor layer is largely unaltered [18, 19]. Although RGCs are lost in *math5* null retinas, mutant cells undergo cell-fate changes [18, 19], making it difficult to unambiguously delineate the effects that RGC might have on the extracellular environment and overall retinal development.

RGCs as a Signaling Source for the Developing Retina

There is a delicate balance during retina development between RPC proliferation and differentiation. Whereas the RGCs promote RPC proliferation through secreted molecules, they also negatively regulate their own production through the same or different secreted factors. *math5*-expression upregulation owing to RGC ablation may indicate a response to the decreased RPC proliferation and reflect the disrupted balance between proliferation and differentiation. The RGC-secreted molecules function to ensure that a sufficient RPC supply is available for specification and differentiation of the later cell types in retinal development.

Supplemental Data

Detailed Experimental Procedures and a supplemental figure are available at http://www.current-biology.com/cgi/content/full/15/6/525/DC1/.

Acknowledgments

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